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Form Approved
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1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	1994	Journal article

4. TITLE AND SUBTITLE
Cold-induced perturbation of cutaneous blood flow in the rat tail: a model of nonfreezing cold injury

5. FUNDING NUMBERS
PE -61153
PR -MR04120
TA -00B
WU -1058

6. AUTHOR(S)
Thomas JR; Shurtleff D; Schrot J; Ahlers ST

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Naval Medical Research Institute
Commanding Officer
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5607

NOV 1994

8. PERFORMING ORGANIZATION REPORT NUMBER
NMRI 94-55

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Naval Medical Research and Development Command
National Naval Medical Center
Building 1, Tower 12
8901 Wisconsin Avenue
Bethesda, Maryland 20889-5606

10. SPONSORING/MONITORING AGENCY REPORT NUMBER
DN240517

11. SUPPLEMENTARY NOTES
Reprinted from: Microvascular Research 1994 Vol.47 pp. 166-176

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution is unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

14. SUBJECT TERMS
nonfreezing cold injury; blood flow; laser doppler flux

15. NUMBER OF PAGES
11

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

MICROVASCULAR RESEARCH 47, 166–176 (1994)

Cold-Induced Perturbation of Cutaneous Blood Flow in the Rat Tail: A Model of Nonfreezing Cold Injury

JOHN R. THOMAS, DAVID SHURTLEFF, JOHN SCHROT, AND STEPHEN T. AHLERS

Thermal Stress Program, Naval Medical Research Institute, Bethesda, Maryland 20889-5607

Received November 17, 1992

Cold-induced alteration of cutaneous blood flow, measured with laser Doppler flowmetry, was studied in a rat tail model of nonfreezing cold injury (NFCI). The NFCI-inducing condition consisted of prolonged tail immersion in 1° water. Before exposure to the injury condition, tail blood flow (laser Doppler flux) during brief 3° immersion showed cold-induced cycles of vasoconstriction followed by cold-induced vasodilation (CIVD). Tail temperature exhibited cyclic patterns similar to blood flow in response to cold water immersion. Cold exposures to 1° for 1 or 3 hr induced no systematic change; however, cold exposures of 6 or 9 hr induced profound and long-lasting blood flow and temperature deviations. Following the cold injury condition, CIVD was completely absent and remained absent for several weeks, suggesting that CIVD loss is an important component in development of NFCI. Cold-induced disturbances of cutaneous blood flow in the rat tail consisted of a sequence of distinctive stages analogous to those described in human NFCI. These stages were evidenced initially by several days of reduced blood flow and thermal sensitivity, followed in a week by a hyperemia stage, and later by enhanced vascular and thermal sensitivity. The cutaneous blood flow alterations and sequence of variations following prolonged cold exposure suggest that the rat tail may be a valid model of human NFCI.

INTRODUCTION

Nonfreezing cold injury (NFCI) is a unique syndrome that results from damage to peripheral tissues, usually in the extremities, exposed to cold temperatures for prolonged periods of time (Francis, 1984; Montgomery, 1954). Although numerous nonvascular components are involved in the development and symptomatology of NFCI, alteration in peripheral blood flow in the cutaneous circulation is most often a salient feature (Montgomery, 1954; Ungley and Blackwood, 1942; Ungley *et al.*, 1945). The pathogenesis of NFCI in humans, as revealed by clinical studies, involves rather distinctive stages (Francis and Golden, 1985). The initial stage, following prolonged cold exposure, is evidenced by reduction in peripheral blood flow and lack of sensation, followed by a hyperemic stage, and then followed by intensified thermal sensitivity that may endure for extended time periods (Ungley *et al.*, 1945).

During initial exposure of an extremity to cold, a predominant response is restriction of peripheral blood flow due to sympathetic vasoconstriction. As an exposed extremity continues to cool, the peripheral circulation usually passes

0026-2862/94 $6.00

through a phase of cold-induced vasodilation (CIVD response) (Lewis, 1930, 1931) thought to be a protective mechanism against damage to peripheral tissues. Prolonged cold exposure reduces or even eliminates the prominent CIVD response (Keatinge and Harman, 1980) and this diminishment of the CIVD response has been implicated in the development of NFCI (Francis and Golden, 1985; Montgomery, 1954).

The purpose of the present study was to describe cold-induced changes in blood flow in a whole animal model of NFCI and specifically to examine those changes in peripheral blood flow with laser-Doppler flowmetry (Shepherd and Öberg, 1990). The rat tail was used as a model since evidence suggests the rat tail can develop some of the pathological conditions during and following cold exposure similar to those observed in man (Ahlers *et al.*, 1990; Blackwood and Russell, 1943; Hellstrom, 1974; Peyronnard *et al.*, 1977; Van Orden *et al.*, 1990). Additionally, CIVD has been found to be most pronounced in cutaneous areas rich in arteriovenous anastomoses (Grant and Bland, 1931; Fox and Wyatt, 1962), structures well represented in the tail of the rat (Gemmell and Hales, 1977; Henningsen, 1969). Importantly, the rat tail clearly demonstrates normal cycles of CIVD during cold water exposure (Eide, 1976; Hellstrom, 1974).

METHOD

The subjects were male Long–Evans rats (Charles River Laboratories) weighing approximately 300 g and individually housed in hanging home cages in an air-conditioned unit with food (NIH Open Formula Chow, Agway Inc.) and water available continuously. Subjects were maintained on a 12-hr light/dark cycle which began with lights on at 6:00 a.m. Housing room temperature was maintained at 25°.

Rats were placed into a ventilated plastic cylindrical holder that allowed the tail to protrude through one end. The holder was affixed to a frame at a 55° angle, allowing the tail to be immersed into water located directly below the holder. Details of the exposure equipment is described elsewhere (Thomas *et al.*, 1992). The protruding rat tail was placed into a 4-cm-long porous plastic cylinder lined with foam rubber and joined to the end of the rat holder. A laser–Doppler blood perfusion probe was attached on the inside of the tail cylinder, allowing the probe to be gently held directly adjacent to the ventral surface of the tail, approximately 14 cm from the tip, and to be located in the same position on subsequent exposures. The probe was a right-angle prism probe (Model PR-434, TSI Incorporated) connected via a fiberoptic cable to a laser–Doppler blood perfusion monitor (Laserflo BPM, Model 403A, TSI Incorporated). During each session a thermocouple was attached with surgical tape to the dorsal tail surface approximately 19 cm from the tip, which located the thermocouple just above the water level when the tail was immersed.

Rats were adapted to the holder over several weeks, until the data of 8 to 10 consecutive 40-min sessions indicated no consistent trend in blood flow (laser–Doppler flux) or temperature. During the week following attainment of baseline stability, each subject was placed in the holder and data recorded for 40 min each day for 5 consecutive days (M–F). On alternate days (M, W, F) the tails of the rats were immersed in 3° cold water in order to obtain data on the normal response

of tail blood flow and temperature to cold before exposure to conditions that might actually induce cold injury. After several initial minutes of blood flow and temperature stabilization, cold water exposure was accomplished by immersing all but 1.5 cm of the tail, for the duration of a session. The water temperature was maintained by a large copper tube coil that surrounded the tail underwater and was connected to a constant-temperature water bath. A small paddle at the bottom of the water container gently stirred the water allowing for the measurement of rat tail blood flow with minimal water movement, as the arrangement of holding the blood flow probe to the tail, while extremely reliable, was exceedingly sensitive to movement artifacts. On the other 2 days of the week (T, TH) the same immersion procedure was followed with the water at 26°. Room temperature was maintained at 25° and temperature, measured with a thermocouple located within the rat holder, w

On the following Mon[illegible] at was placed in a plastic holder, similar to the one used during baseline and the tail was immersed in 1° water. To distinguish this cold exposure from those of shorter duration, this condition will be referred to as the "injury condition." In order to measure the magnitude of possible cold-induced injury as a function of length of exposure, rats were exposed once to 1, 3, 6, or 9 hr of cold. Specifically, 6 rats were exposed to the injury condition for 1 hr, 10 rats were exposed for 3 hr, 18 rats were exposed for 6 hr, and 10 rats were exposed for 9 hr. Moreover, as a control procedure other rats were exposed with the water maintained at 26°C. Under the control conditions, 8 rats were exposed for 6 hr and 6 rats were exposed for 9 hr. Control rats were not exposed for 1 or 3 hr, as results of exposure to the injury condition at these times did not indicate any systematic cold-induced change in tail blood flow or temperature. The longer cold sessions involved exposure of only the tail, with minimal direct thermal stress to the whole animal. Nine hours of restraint exposure did not appear to induce any severely stressful impact on the subjects, as the holder did allow for movement and the control animals appeared rather normal following removal from the holder.

Following exposure to one of the above injury conditions, blood flow and temperature were monitored over a 2½-week interval to obtain data on any injury condition-induced changes in response to cold as follows: on postinjury condition Days 1, 3, 8, 10, and 15, the tail of the rats were exposed to 26° water in an identical manner to that during the week prior to the injury condition. On post injury condition Days 2, 4, 9, 11, and 16, the tails of rats were exposed to a 40-min 3° temperature water condition identical to that during the week prior to the injury condition.

The laser–Doppler and thermocouple outputs were connected to a computer-controlled system via an A/D converter. The blood flow and temperature channels were sampled and recorded every second and subsequently averaged over 15-sec intervals for further data analysis. Summary results are presented as means ± SEM. Significant differences between measures were assessed using repeated measures analysis of variance (ANOVA). The least square means multiple comparison test was used to determine differences among paired comparisons.

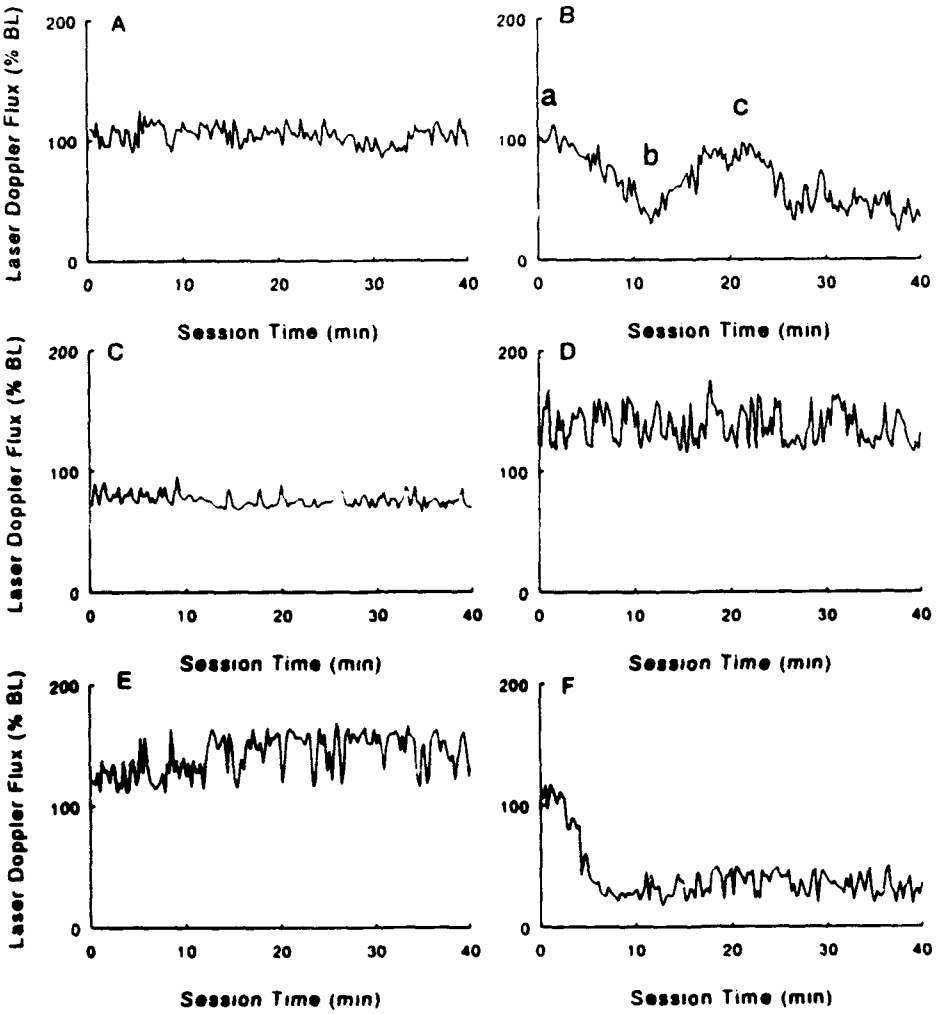

FIG. 1. Rat tail blood flow (laser-Doppler flux) records (plotted as percentage of baseline) of entire experimental sessions for (A) baseline session of tail immersion in 26° water, (B) tail immersion in 3° water prior to injury condition, (C) tail immersion in 3° water on 2nd day postinjury condition, (D) tail immersion in 26° water on 8th day postinjury condition, (E) tail immersion in 3° water on 9th day postinjury condition, and (F) tail immersion in 3° water on 16th day postinjury condition.

RESULTS

Tail blood flow. Blood flow (laser-Doppler flux) recorded during the last three baseline sessions was averaged over each 40-min session, and the mean of these sessions was used as the baseline value for each rat. Overall, reproduction of the laser-Doppler measurements was about 10%. All further blood flow data for each rat were calculated as a percentage of that baseline mean. Figure 1A shows a record of the blood flow output obtained during a baseline session for a representative rat.

Figure 1B shows the blood flow of a representative rat during a 40-min cold session prior to the injury condition. Typically, after the initial decline, the tail exhibited a single cycle of blood flow lasting about 15–20 min. On several oc-

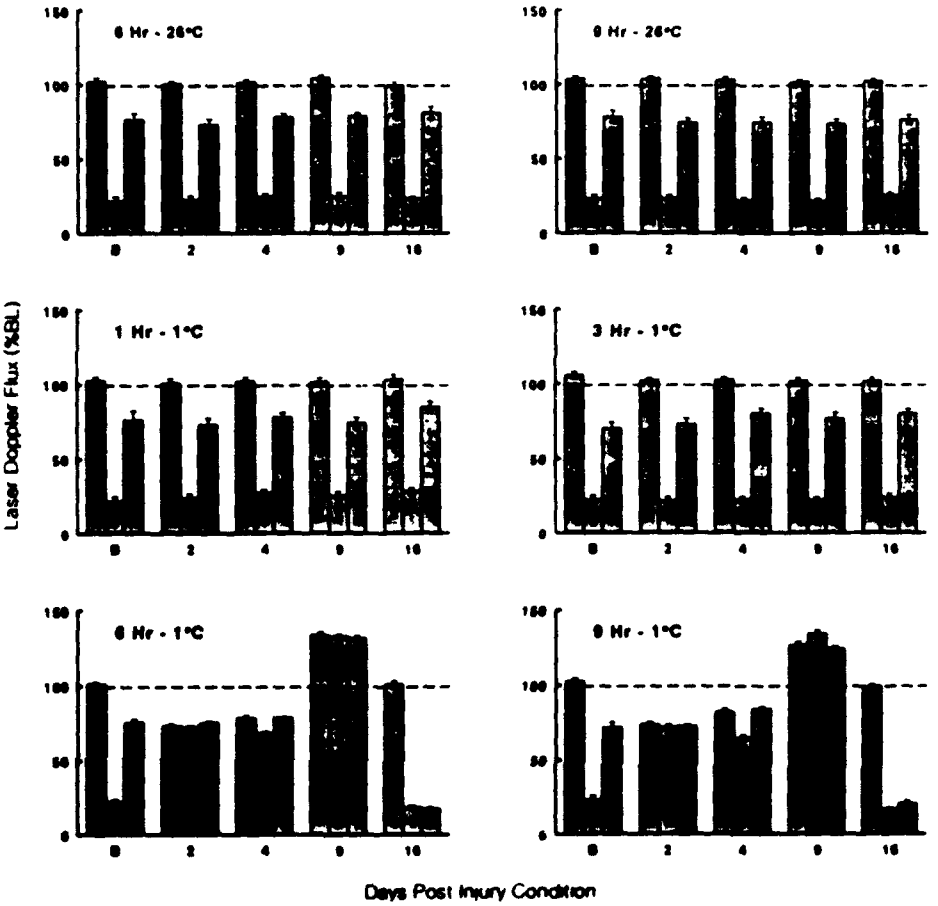

FIG. 2. Summary data for tail blood flow (means ± SEM) for baseline (B) and several cold exposure sessions for each of six exposure conditions. In each triad, the left bar represents initial values on cold exposure days prior to tail immersion, the middle bar is the lowest value obtained during tail immersion, and the right bar indicates the peak value obtained after the initial cold-induced decline. When no clear decline occurred during immersion, data were taken from the temporal position within a session prior to the injury condition where each subject normally displayed asymptotic decline and where the peak increase following an initial decrease in blood flow was normally observed.

casions, however, blood flow displayed the beginning of a second cycle within the final 10 min of the session, or showed two full cycles, each about 10 min in duration. Labels a, b, and c represent the blood flow measures used for analysis and correspond to the leftmost, middle, and rightmost value of the triads shown in Fig. 2, respectively. The leftmost value of the leftmost triad in each section of Fig. 2 represents blood flow just prior to cold immersion, the middle bar represents the lowest blood flow at the end of the initial decline following tail immersion, and the rightmost bar of the triad displays peak blood flow during the increase after the initial decline. Under conditions in which blood flow did not evidence this baseline pattern, blood flow at the approximate corresponding baseline time interval was used. As illustrated in Fig. 2, this pattern of blood flow significantly changed as a function of exposure session and injury condition. Consequently,

ANOVA analysis revealed a significant Injury Condition × Cold Exposure Session × Triad interaction ($F(40, 448) = 39.21$; $P < 0.01$) for blood flow.

During the baseline exposure session, prior to the injury condition, all rats for all groups (Fig. 2, leftmost triads labeled "B") showed a blood flow pattern similar to that shown in Fig. 1B. Paired comparisons indicated that the lowest blood flow (middle bar of triad) was significantly lower than initial blood flow (all Ps < 0.01 for all groups). The subsequent increase in blood flow (right bar of triad), although significantly greater than the lowest blood flow measure, was significantly lower than baseline levels for all groups (all Ps < 0.01), indicating that, although blood flow increased in the later part of a session, it did not reach preimmersion levels.

Over subsequent exposure sessions following injury, the pattern of blood flow changed as a function of exposure condition. Blood flow of control rats exposed to 26° water for either 6 or 9 hr (Fig. 2, top sections) evidenced no significant change in the pattern of blood flow over the subsequent cold exposure test sessions. Blood flow for rats exposed to 1° water for either 1 or 3 hr (Fig. 2, middle sections) also evidenced no change in the pattern of blood flow over the subsequent cold exposure test sessions, relative to baseline.

Tail blood flow in rats exposed to 1° water for either 6 or 9 hr did manifest clear systematic changes over cold postinjury test sessions relative to baseline. On the second day following injury the 6- and 9-hr cold injury groups showed a significant reduction in blood flow of about 20 to 25% in the initial portion of the triad prior to cold water immersion, relative to all other groups (all Ps < 0.01), and their respective baseline levels (both Ps < 0.01). Blood flow remained constant throughout the session such that blood flow measures for the second and third portions of the triad were not different from each other (both Ps > 0.20) or the initial blood flow measure (both Ps > 0.20). Figure 1C is a representative blood flow record for a rat during immersion of the tail in 3° water on the second day postinjury condition. This pattern of blood flow for the 6- and 9-hr groups was also evident during the fourth day postinjury.

By the eighth day postinjury condition (26° day), blood flow was 20–25% higher than normal. Figure 1D displays the increased blood flow on Day 8 for a single rat. Figure 1D shows, in addition to higher blood flow, a large increase in the blood flow variability. On Day 9 blood flow was higher than normal at the beginning of the cold exposure session and continued to be higher than normal during cold immersion for 6- and 9-hr groups. Consequently, paired comparisons indicated that blood flow for each value of the triad was significantly higher than their respective baseline levels (all Ps < 0.01). A representative record for a rat tail immersed in cold water on Day 9 is presented in Fig. 1E.

By Day 16 tail blood flow returned to within normal range for the initial value of the triad, relative to baseline for both the 6- and 9-hr cold exposure groups (both Ps > 0.10). A clear blood flow response to cold on this day was evidenced by a significant decline following immersion, as illustrated by the middle value of the triad for both groups. Paired comparisons indicated that this decline in blood flow was significantly lower than the first value of the triad on this day for both groups (both Ps < 0.01). During the last portion of the cold exposure test session blood flow remained low and did not increase. A representative tail blood flow record during cold water immersion for Day 16 postinjury condition is displayed in Fig. 1F.

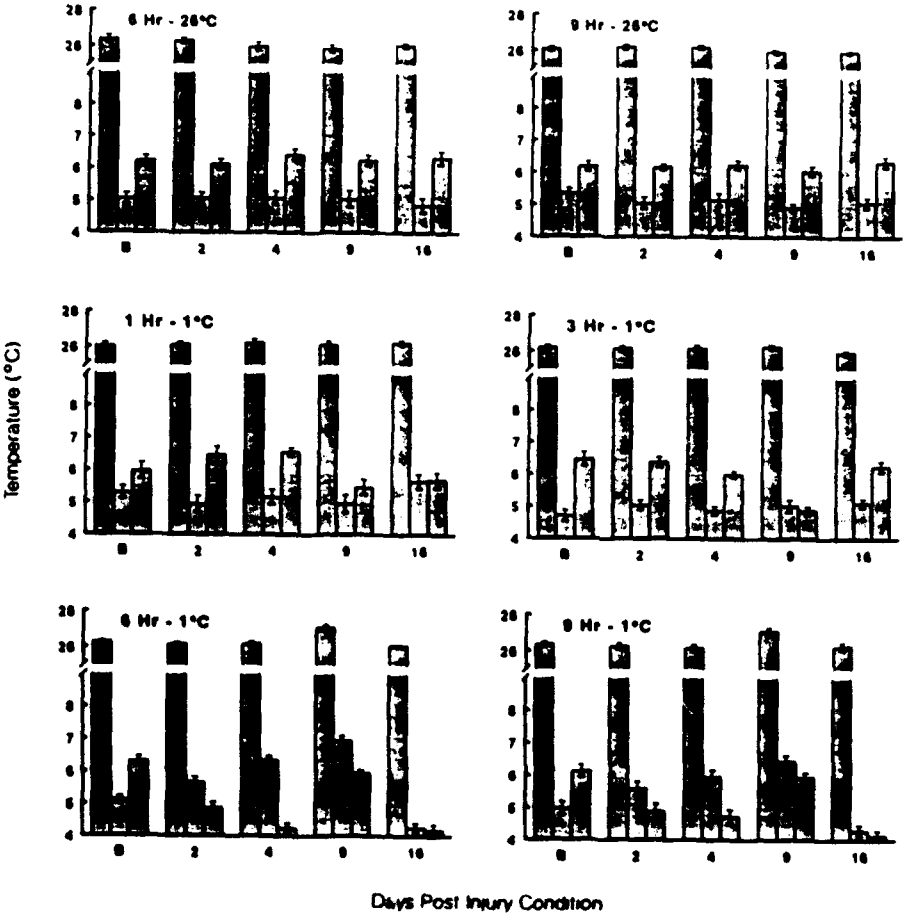

FIG. 3. Summary data for tail temperature (means ± SEM) for baseline (B) and several cold exposure sessions for each of six exposure conditions. Data format is identical to that of Fig. 2.

Tail temperature. The leftmost triad in each panel of Fig. 3 represents baseline tail temperature. Tail temperature essentially followed the same pattern as blood flow during cold water immersion before exposure to the injury condition (leftmost triads labeled "B"). Following the injury condition the pattern of tail temperature changed as a function of injury condition and cold exposure session. Consequently, as with tail blood flow, ANOVA analysis revealed a significant Injury Condition × Cold Exposure Session × Triad interaction ($F(40, 448) = 7.33$; $P < 0.01$) for tail temperature. During baseline cold exposure sessions tail temperature significantly declined from approximately 26.0° (left bar) to about 5° (middle bar) for all groups (all Ps < 0.01) and then increased significantly by about 1° (right bar) for all groups (all Ps < 0.01), relative to the middle tail temperature value.

Over subsequent exposure sessions following injury, the pattern of tail temperature exhibited changed as a function of exposure condition. Control rats exposed to 26° water for either 6 or 9 hr evidenced no change in the pattern of tail temperature change over the subsequent cold exposure test sessions. For the 1- and 3-hr cold injury groups, tail temperature did not change across postinjury cold test sessions relative to preinjury baseline.

For the 6- and 9-hr cold exposure groups the pattern of tail temperature change following the cold injury condition differed significantly from baseline. The preimmersion tail temperature (first value of the triad) was the same as baseline levels on postinjury test Days 2, 4, and 16 (all $Ps > 0.25$). However, on test Day 9 tail temperature was significantly higher for both groups than their respective baseline levels (both $Ps < 0.01$). Tail temperature for the middle value of each triad, following cold injury, for both groups was significantly different from baseline. On cold test Days 2, 4, and 9 tail temperatures were significantly higher than baseline (all $Ps < 0.01$), while on test Day 16 tail temperatures were significantly lower than baseline (both $Ps < 0.01$) for the middle value of the triad. Tail temperature for the rightmost value of the triad was significantly lower than baseline levels on all postinjury test days for both 6- and 9-hr groups (all $Ps < 0.01$).

DISCUSSION

Before exposure to the injury condition, the cutaneous blood flow of the rat tail, measured with laser–Doppler flowmetry, showed consistent cold-induced cyclic or multicyclic epochs consisting of reduced blood flow followed by increased blood flow. The cyclic changes, elicited by immersion of the rat tail in 3° water, are strongly indicative of the phenomenon of CIVD, appear qualitatively similar to CIVD reported in humans (Lewis, 1930, 1931), and display considerable pattern variability as also observed in humans. Moreover, these cyclic patterns appear comparable to patterns of cold-induced vasodilatation reported in an *in vitro* preparation of isolated segments of rat tail arteries exposed to cold (Gardner and Webb, 1986). That study suggested that the control of initial cold-induced vasoconstriction is associated with norepinephrine release, and the cold-induced vasodilatation phase is related to a cold-modulated cessation of norepinephrine release. Other studies have similarly suggested that CIVD cycles result from such norepinephrine modulation (Shepherd *et al.*, 1983).

CIVD has previously been assessed in the rat tail by changes in tail temperature (Eide, 1976; Hellstrom, 1974). In those studies, after initial decrease produced by immersion in ice water, tail temperature increased followed by further cycles after varying time periods. The maximal tail temperature of the first cold-induced vasodilation and the time at which peak temperature was reached, although somewhat variable, was comparable in those studies to the temperature variations observed in the present study. The temperature measures of CIVD in the rat tail observed in those studies were also similar in appearance and temporal patterning to cold-induced blood flow changes in the present study. Importantly, in the present study the CIVD response was completely absent following prolonged exposure to the cold injury condition and remained absent for at least several weeks after exposure to the injury condition, implicating the loss of CIVD, as previously suggested, as a significant component in the development of NFCI (Francis and Golden, 1985, Montgomery, 1954).

Cold-induced changes were demonstrated to be a function of length of cold water exposure. No significant vascular or temperature changes were induced by 1 or 3 hr exposure, but significant changes did occur after 6 or 9 hr. In a similar fashion, research focused on histological changes in experimentally produced NFCI

found cold-induced injury in muscle and nerves of the rat tail varied with the length of cold water exposure (Blackwood and Russell, 1943). These findings are consistent with observations in humans that some minimum time of exposure to cold temperatures is required for an injury to become apparent (Meryman, 1957).

The present study discloses that the cutaneous blood flow in the rat tail following prolonged cold water exposure clearly exhibits distinctive phases reflective of many of the characteristic patterns described in humans with NFCI (Francis and Golden, 1985; Ungley and Blackwood, 1942; Ungley *et al.*, 1945). Over the first few hours to days following prolonged cold exposure in humans, referred to as the Post-Exposure Stage (Ungley *et al.*, 1945), the exposed extremity usually exhibits diminished circulation. During the first few days following exposure to the injury condition, blood flow of the rat tail was lower than before the exposure, and the tail did not exhibit a clear response to cold water immersion, in that there was no cold-induced decrease in blood flow. Disruptions observed in rat tail blood flow during the first week after exposure to the injury condition parallel those changes observed in the human Post-Exposure Stage.

Following the Post-Exposure Stage in humans is a Hyperemia Stage (Ungley *et al.*, 1945) often lasting from days to months and characterized by increased blood flow and often loss of reflexive vasoconstriction. In the present study, the initial lowered blood flow, observed during the first week, was replaced in the second week with one of blood flow notably increased above baseline and associated with enhanced flow variability. During this time the tail still did not evidence blood flow decline in response to cold water exposure, but displayed increased variability with blood flow constantly maintained above preinjury condition levels. This configuration of changes appears functionally similar to the Hyperemia Stage in humans.

The Post-Hyperemia Stage in humans has a duration of days to months to years and is associated with thermal hypersensitivity and a delay or impairment of reflexive vasomotor responses (Ungley *et al.*, 1945). By Day 15 postinjury condition, the peripheral blood flow in the tail returned to near normal flow level. During cold immersion on Day 16 the tail again displayed a decline in blood flow, but the decline appeared to be a more rapid and greater drop in flow than during preinjury condition exposures, perhaps an indication of enhanced sensitivity analogous to symptoms described in the Post-Hyperemia Stage in humans. Additionally, on Day 16 the temperature of the tail dropped more rapidly and reached lower levels than before the injury condition exposure and may also be reflective of enhanced thermal sensitivity of the rat tail.

Numerous animal studies have focused on vascular and neural dysfunctions associated with exposures to nonfreezing cold (for example, Blackwood and Russell, 1943; Das *et al.*, 1991; Denny-Brown *et al.*, 1945; Endrich *et al.*, 1990; Gilliatt and Kennett, 1987; Nukada *et al.*, 1981; Peyronnard *et al.*, 1977); however, with the exception of the Blackwood and Russell, study results of those animal studies are not necessarily pertinent to the unique human NFCI condition as they do not indicate a discernible progression of stages following cold exposure analogous to the syndrome described in humans. Taken together with data from other cold injury studies employing the rat tail model, which show that the tail exhibits distinctive stages of loss of thermal sensitivity followed by enhanced thermal sensitivity (Ahlers *et al.*, 1990) and manifests differential stages of characteristic

neural dysfunctions (Shurtleff *et al.*, 1993; Van Orden *et al.*, 1990), the perturbations in cutaneous blood flow observed in the present study suggest that the rat tail may be an extremely functional and valid model of human NFCI.

ACKNOWLEDGMENTS

This research was supported by Naval Medical Research and Development Command work unit 61153N.MR04120.00B.1058. The opinions and assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large. The experiments reported herein were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals." Institute of Laboratory Animal Resources, National Research Council, DHHS Publication (NIH) 86-23, 1985.

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